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RESEARCH PAPER

Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*

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Abstract

In this study the interplay between the symbiotic and defence signalling pathways in *Lotus japonicus* was investigated by comparing the responses to *Mesorhizobium loti*, the symbiotic partner of *L. japonicus*, and the elicitor flg22, a conserved peptide motif present in flagellar protein of a wide range of bacteria. It was found that defence and symbiotic pathways overlap in the interaction between *L. japonicus* and *M. loti* since similar responses were induced by the mutualistic bacteria and flg22. However, purified flagellin from *M. loti* did not induce any response in *L. japonicus*, which suggests the production of other elicitors by the symbiotic bacteria. Defence responses induced by flg22 caused inhibition of rhizobial infection and delay in nodule organogenesis, as demonstrated by the negative effect of flg22 in the formation of spontaneous nodules in the *snf1 L. japonicus* mutant, and the inhibition of *NSP1* and *NSP2* genes. This indicates the antagonistic effect of the defence pathway on the nodule formation in the initial rhizobium–legume interaction. However, the fact that flg22 did not affect the formation of new nodules once the symbiosis was established indicates that after the colonization of the host plant by the symbiotic partner, the symbiotic pathway has prevalence over the defensive response. This result is also supported by the down-regulation of the expression levels of the flg22 receptor FLS2 in the nodular tissue.

Key words: Defence, flg22, FLS2, *Lotus japonicus*, *Mesorhizobium loti*, symbiosis.

Introduction

Plants of the legume family can benefit from atmospheric nitrogen fixation by forming symbioses with a diverse group of nitrogen-fixing soil bacteria known as rhizobia. This symbiotic interaction is characterized by the formation of root nodules, a specialized plant organ that provides an optimized environment for the bacteria to reduce atmospheric nitrogen into ammonia. Symbiotic interactions between rhizobia and legumes are initiated by an exchange of molecular signals between the host plant and its bacterial symbiont (Perret *et al.*, 2000). The best known bacterially derived signals are the Nod factors (NFs), that consist of a chitin oligosaccharide backbone, in which the non-reducing ends are *N*-acylated and the reducing ends are modified by various molecules (Cullimore *et al.*, 2001; Bek *et al.*, 2010). The structure of NFs varies between rhizobial species and is crucial in determining their host specificity. Application of NFs at nanomolar to picomolar concentrations elicits root hair deformation and nodule primordium formation, and induces the expression of early nodulin genes in host plants (Minami *et al.*, 1996; Niwa *et al.*, 2001), indicating that NFs are key signal molecules triggering legume–rhizobium symbiosis. Putative NF receptors have been identified in various legumes including NFR1 and NFR5 from *Lotus japonicus* (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). In *L. japonicus*, mutations of *LjNFR1* and *LjNFR5* completely abolish the symbiotic responses upon inoculation with *Mesorhizobium loti* or application of purified NFs, suggesting that LjNFR1 and LjNFR5 act co-ordinately for the specific recognition of NFs produced by *M. loti* (Radutoiu *et al.*, 2007). These

putative NF receptors are closely related to the chitin receptor CERK1 in *Arabidopsis* (AtCERK1) (Miya *et al.*, 2007) involved in the recognition of microbial signal molecules termed elicitors or, more recently, microbe-associated (or pathogen-associated) molecular patterns (MAMPs/PAMPs) (Mackey and McFall, 2006). This recognition results in the so-called PAMP-triggered immunity (PTI) that induces defence systems to prevent invasion by hostile microbes (Boller and Felix, 2009). Recently, the evolutionary relationship between the symbiotic and defensive signalling processes has been demonstrated based on the finding that limited alterations in the chitin receptors are crucial for the shift of the intracellular signalling from defence to symbiosis (Nakagawa *et al.*, 2011). In order to investigate the interplay between symbiotic and defence pathways in *L. japonicus*, flagellin, a well-known MAMP of plant pathogens recognized by most plant species, was used. The peptide flg22, corresponding to the highly conserved N-terminal part of flagellin, acts as a potent elicitor in most plant species and is as active as the full-length flagellin (Felix *et al.*, 1999). Flagellin is recognized by a leucine-rich repeat (LRR) receptor kinase FLS2, first identified in *Arabidopsis thaliana* (Gomez-Gomez and Boller, 2000; Chinchilla *et al.*, 2007), although FLS2 orthologues have been cloned in tomato, *Nicotiana benthamiana*, and rice (Hann and Rathjen, 2007; Robatzek *et al.*, 2007; Takai *et al.*, 2008). Flagellin has been portrayed as an invariant MAMP in plants, although data are accumulating to suggest that variation occurs within species as well within pathovars, limiting the defence-eliciting activity of flagellin (Sun *et al.*, 2006). One example would be flagellins of plant-associated bacteria such as *Agrobacterium* and the rhizobia that are exceptionally divergent in the N-terminal domain and are completely inactive as elicitors in different plant species (Felix *et al.*, 1999). The FLS2–flg22 interaction leads to increased intracellular Ca^{2+} concentration, oxidative burst, activation of mitogen-activated protein kinases (MAPKs), transcription of defence-related genes through the WRKY transcription factors WRKY22/29 and WRKY25/33, and ethylene biosynthesis, among other effects (Nicaise *et al.*, 2009). Interestingly, many of these responses have also been detected in epidermal cells of legume roots in response to NF application (Ramu *et al.*, 2002), or inoculation with compatible rhizobia, with the difference that the defence responses detected are transient and local (Cárdenas *et al.*, 2008). Indeed, it has been proposed that plant defence-like responses might be involved in a successful establishment of the symbiotic interaction (D'Haeze *et al.*, 2003), suggesting that similar strategies have been acquired by pathogenic and mutualistic bacteria to establish compatible associations with their host plants. All these findings highlight the interest in investigating PTI in the establishment of the rhizobium–legume symbiosis, and in particular the interplay between symbiotic and defence pathways in plants.

In this study, the defence and symbiotic responses in *L. japonicus* roots in response to its symbiotic partner *Mesorhizobium loti* and the elicitor flg22 were investigated. The effect of PTI on the symbiotic pathway was studied by determining the expression of symbiosis-related genes in plants co-treated with NFs and flg22, and it was found that PTI had a negative effect on nodule formation and establishment of symbiosis.

Materials and methods

Plant material and growth conditions

The *L. japonicus* plants used in this study are in the ecotype Gifu B-129 background (Handberg and Stougaard, 1992). Seeds were scarified by immersion in concentrated H_2SO_4 for 5 min, washed with sterile water, surface sterilized by immersion in 5% NaClO plus Tween-20 for 20 min, and germinated on 1.0% water–agar plates at 28 °C in the dark. Plants were grown in a controlled environmental chamber with a 16/8 h light/dark cycle, 22/18 °C day/night temperature, and relative humidity 65%/75%.

Cloning of LjFls2 *cDNA*

The *L. japonicus* ecotype MG-20 *Fls2* gene (accession no. JN099749) was found in the sequences from the Kazusa DNA Research Institute, Japan by BLAST search using the second exon of the *Arabidopsis FLS2* gene. This gene containing one intron is present on *L. japonicus* TAC clone LjT25G24. Marker TM2139 shows that this clone is located on chromosome 4 at position 8.4 cM (see http://www.kazusa.or.jp/lotus/clonelist4.html). Nine *LjFls2* cDNA clones were found in the expressed sequence tag (EST) database at the National Centre for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov) (Supplementary Table S2 available at *JXB* online). Most of these clones were subsequently fully sequenced. An almost full-length root cDNA clone still containing the intron (MRL034f06, accession no. BP085403.1 giving the 3′ end sequence) was extended by PCR with a long primer (5′-CACCATGTTATCTCTAAAGTTTAGTTTGACTC-TGGTCATAGTC-3′) to obtain a full-length cDNA for cloning in the pENTR vector. Four full-length cDNA clones without the intron were also found. The sequences of the two longest full-length clones are identical to that of the gene, apart from two base changes giving two changed amino acids in the LRR domain (E230 in MG-20 to K and G246 to E). The full-length cDNA sequence of the longest clone has the accession no. JN099750. Five different poly(A) addition sites were found in the various cDNA clones.

Gene expression analyses

For expression analysis of the *FLS2* gene in plant organs, *L. japonicus* wild type and the spontaneous nodulating mutant *snf1* were grown in soil (vermiculite) supplied with nitrogen-free B&D medium (Broughton and Dilworth, 1971) and only the wild-type plants were inoculated with *M. loti* MAFF303099. Five weeks after rhizobial inoculation, the various organs (leaf, root, and nodules) were collected. In order to perform defence and nodulation gene expression analyses in plant roots, 10 *L. japonicus* wild-type plants were grown for 4 weeks in square Petri dishes on solid 1/4 B&D medium without nitrate. The roots were shielded from light by wrapping the lower half of the plate with a black cover. To study defence gene expression, each root was treated with 100 µl of a solution of 1.0 µM flg22 and/or a cell suspension of *M. loti* (OD_{600} ~0.02) for 1 h. Mock treatment consisted of water. For nodulation gene expression analysis, each root was treated with 100 µl of a solution containing 1.0 µM flg22 and/or 0.1 µM NF for 24 h. Total RNA was extracted using an RNeasy plant mini kit (Macherey-Nagel, Düren, Germany) followed by treatment with RNase-free DNase I (Ambion, Austin, TX, USA) for genomic DNA removal. First-strand cDNA was synthesized

using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA) from 0.3 μg of total RNA. The cDNA samples were tested for contaminating genomic DNA using PCR primers specific for the *Nin* promoter. Real-time reverse transcription-PCR (RT-PCR) was performed using Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) and 1 μl of a 2-fold diluted cDNA template. Results were quantified using the ΔΔCt method (Livak and Schmittgen, 2001). Transcript levels were normalized to *LjATPsynthase* (Czechowski *et al.*, 2005). Primer sequences for quantitative RT-PCR analysis are provided in Supplementary Table S1 at *JXB* online.

Nodulation kinetics and nitrogenase activity

Lotus japonicus wild type and *snf1* were grown in square Petri dishes on solid 1/4 B&D medium without nitrate for nodulation kinetics. Wild-type plants were inoculated with *M. loti* MAFF303099 (OD_{600} ~0.02) 2 d after transfer to the Petri dishes, and the nodule number was monitored during 5 weeks. For the acetylene reduction assays, plants were grown in 50 ml Leonard jars filled with vermiculite. Twelve plants were used at each time point and the nodule number was also determined.

Ethylene production assay

For ethylene production assays, 5-day-old *L. japonicus* seedlings were used. Ethylene biosynthesis was assayed by placing two seedlings in 6 ml tubes with 100 μl of water or water containing 0.5 μM flg22 or a cell suspension of *M. loti* (OD_{600} ~0.02). Tubes were sealed with rubber caps, and ethylene accumulating in the headspace within 24 h of incubation was determined by gas chromatography.

MAPK assay

MAPK assays were performed on roots of 4-week-old *L. japonicus* plants growing in vermiculite. The roots were cut into 0.5 cm pieces and floated in Petri dishes overnight at room temperature. For each treatment, 75 mg of roots were elicited for 15 min with 0.5 μM flg22, a cell suspension of *M. loti* (OD_{600} ~0.5), or purified flagellin from *M. loti* at a protein concentration determined by the Bradford protein test equivalent to the concentration of flg22. MAPK activation was monitored by western blot with antibodies that recognize the dual phosphorylation of the activation loop of MAPK (pER-K, Cell Signaling Technology, USA). Blots were stained with Ponceau red to verify equal loading.

Isolation of flagellin from M. loti

Cells of *M. loti* MAFF303099 were grown in TY medium in an orbital shaker (180 rpm) at 28 °C for 48 h. Cells were harvested by centrifugation at 10 000 *g* for 20 min and resuspended in 20 mM TRIS pH 7.0. Cells were deflagellated in a blender (Waring Model 607-3) twice for 30 s. The flagella were separated from the cell bodies by centrifugation at 10 000 *g* for 20 min and were concentrated from the supernatant by centrifugation at 100 000 *g* for 30 min. The pellet was resuspended 1 ml of H_2O, the pH was adjusted to 2.5 with HCl, and boiled for 10 min to dissociate flagella from the monomeric flagellin subunits, followed by centrifugation at 100 000 *g* for 30 min to remove acid-insoluble impurities. The pH of the supernatant containing flagellin was adjusted back to pH 7.0 with NaOH. The purity of the flagellin preparation was determined by SDS–PAGE using a 12% gel and proteins were visualized by Coomassie blue staining (see Supplementary Fig. S1 at *JXB* online).

Results

Mesorhizobium loti *and flg22 induce similar defence responses in* L. japonicus

The effect of flg22 on different defence responses was tested in *L. japonicus* and compared with the effect of a bacterial suspension of *M. loti*, the symbiotic partner of *L. japonicus*. The production of the defence hormone ethylene in sterile seedlings increased in response to 0.5 μM flg22 (2-fold related to the control) and the bacterial suspension (OD_{600} ~0.02). However, purified flagellin from *M. loti* did not induce any ethylene accumulation (Fig. 1). Phosphorylation of MAPKs as a means of controlling protein activity occurs in diverse processes in eukaryotic cells including plant defence (Colcombet and Hirt, 2008). It was observed that protein extracts from *L. japonicus* roots treated with flg22 and the *M. loti* cell suspension for 15 min contained activated protein kinases with relative molecular masses of ~45 kDa (Fig. 2) which is consistent with the expected size for MAPKs (Asai *et al.*, 2002). The activation level with the *M. loti* bacterial suspension was lower than the response induced by flg22, and purified flagellins from *M. loti* did not induce MAPK phosphorylation (Fig. 2). Expression analysis by quantitative RT-PCR revealed the up-regulation of defence-related genes *Lj MPK3* and *Lj WRKY33* by flg22 and, at a lower but significant level, by the bacterial suspension. However, the expression of *Lj CP450*, involved in the synthesis of camalexin (Nafisi *et al.*, 2007) was similarly induced by flg22 and the symbiotic bacteria (Fig. 3). All those data together suggest that other elicitors different from flagellin must be present in the bacterial suspension inducing the conserved defence signalling pathway.

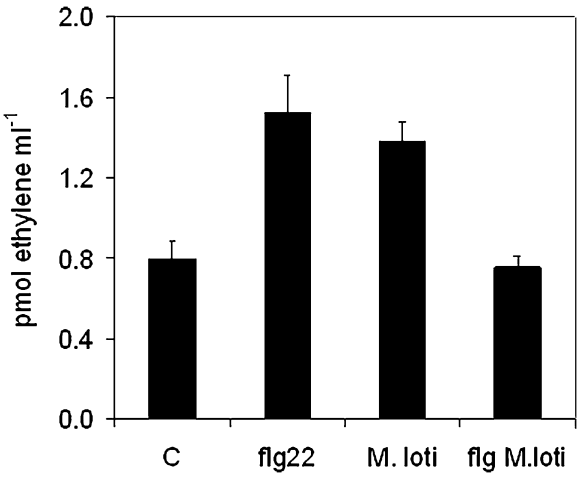

Fig. 1. Induction of ethylene production in seedlings of *L. japonicus* either untreated (C) or treated with 0.5 μM flg22, a cell suspension of *M. loti* (OD_{600} 0.02), and flagellin purified from *M. loti* (flg M. loti) at a protein concentration equivalent to 0.5 μM flg22 for 24 h. Vertical bars represent the average and SE of three independent experiments.

Flg22 inhibits the symbiosis establishment between L. japonicus *and* M. loti

The effect of 0.1 μM and 1.0 μM flg22 on the symbiotic establishment upon inoculation with *M. loti* was analysed by comparing the nodulation kinetics. The nodule number per plant was monitored during 5 weeks after inoculation in sterile conditions. After 1 week, the first nodules became visible in plants growing in control conditions and in the presence of 0.1 μM flg22 (Fig 4A), with a reduction of 68% in the nodule number by the flg22 effect. In plants growing in the presence of 1.0 μM flg22, the first nodules could only be observed 2 weeks after inoculation when the largest differences in nodule number between control and flg22-treated plants were detected. However, the nodule number reached similar values 4 and 5 weeks after inoculation in control and 0.1 μM flg22 conditions, while the plants with 1.0 μM flg22 maintained a 25% lower nodule number. In order to determine whether the negative effect of flg22 in the nodulation was due to an inhibition of the infection process or the nodule organogenesis, the formation of spontaneous nodules in the *L. japonicus* gain-of-function mutant *snf1* encoding a constitutively active calcium/calmodulin-dependent protein kinase (CCaMK) was tested (Fig. 4B). This spontaneous nodulation mutant uncouples organogenesis from infection, leading to the formation of empty spontaneous nodules in the absence of the bacterial symbiont (Tirichine *et al.*, 2006*a*, *b*). *Lotus snf1* plants were grown in the same conditions as wild-type plants but they were not inoculated and the spontaneous nodules formed were monitored for 5 weeks. In control conditions, the first nodules appeared after 3 weeks, while in the presence of 0.1 μM and 1.0 μM flg22, nodules became visible after 4 and 5 weeks, respectively (Fig. 4B). In addition, the average nodule number in the presence of flg22 was 80% lower than in the control.

To confirm the effect of flg22 in the nodule initiation, transcription factor nodulation signalling pathway (*NSP*) and early nodulin *ENOD40* genes, involved in rhizobial infection and nodule organogenesis, were subjected to quantitative RT-PCR analysis (Fig. 5). In this experiment, purified NF from *M. loti* R7A instead of a bacterial suspension was used to induce nodulation in order to evaluate the effect of flg22 without interference from bacterial elicitors. Roots of *Lotus* growing in sterile conditions were subjected to treatments with 1.0 μM flg22 and/or 0.1 μM NF for 24 h. RT-PCR revealed that treatment with flg22 caused a slight decrease in the transcript level of *NSP1* and a stronger decrease (by 70%) in the transcript level of *NSP2* and *ENOD40*. In contrast, as expected, the levels of *NSP1*, *NSP2*, and *ENOD40* transcripts increased with the NF treatment between 2- and 4-fold. Interestingly, the co-treatment with NF and flg22 reduced the expression level of the three mentioned genes to levels similar to the control, or even lower in the case of *ENOD40*. The *NIN* gene, involved in infection threat formation and nodule primordial initiation, showed a different expression pattern: both flg22 and NF induced *NIN* gene expression, and their effect appeared to be additive.

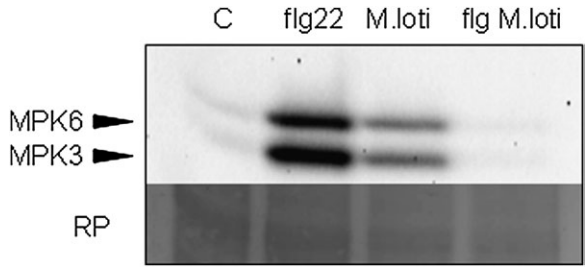

Fig. 2. Activation profile of MAPKs in roots of *L. japonicus* in response to treatments with flg22 (0.5 μM), a cell suspension of *M. loti* (OD_{600} 0.5), and flagellin purified from *M. loti* (flg M.loti) at a protein concentration equivalent to 0.5 μM flg22 for 15 min. Activated MAPKs were detected by western blot with antibodies that recognize the dual phosphorylation of the activation loop of MAPKs. Arrowheads indicate phosphorylated MAPK3 and MAPK6. Blots stained with Ponceau red (PR) are presented to show equal loading.

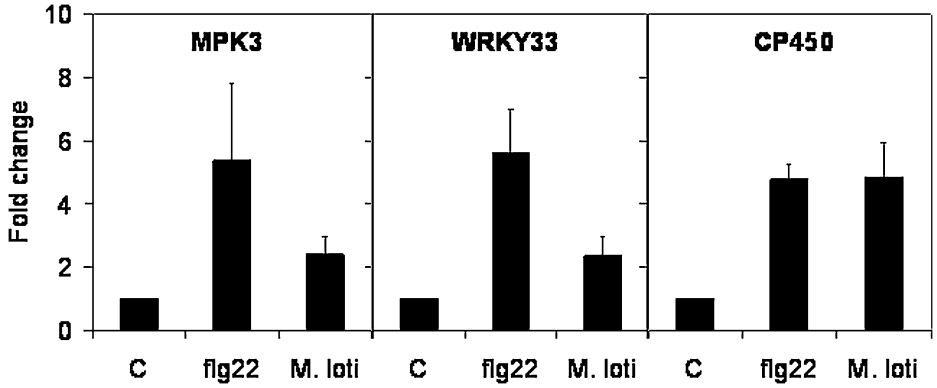

Fig. 3. Quantitative RT-PCR analysis of *MPK3*, *WRKY33*, and *CP450* transcripts in roots of *L. japonicus* untreated (C), or treated with 1.0 μM flg22 and with a cell suspension of *M. loti* (OD_{600} 0.02) for 1 h. Expression levels are normalized to the housekeeping gene *ATP synthase* and are presented as relative to untreated plants. The data are expressed as the means and SE of two biological replications of 10 plants.

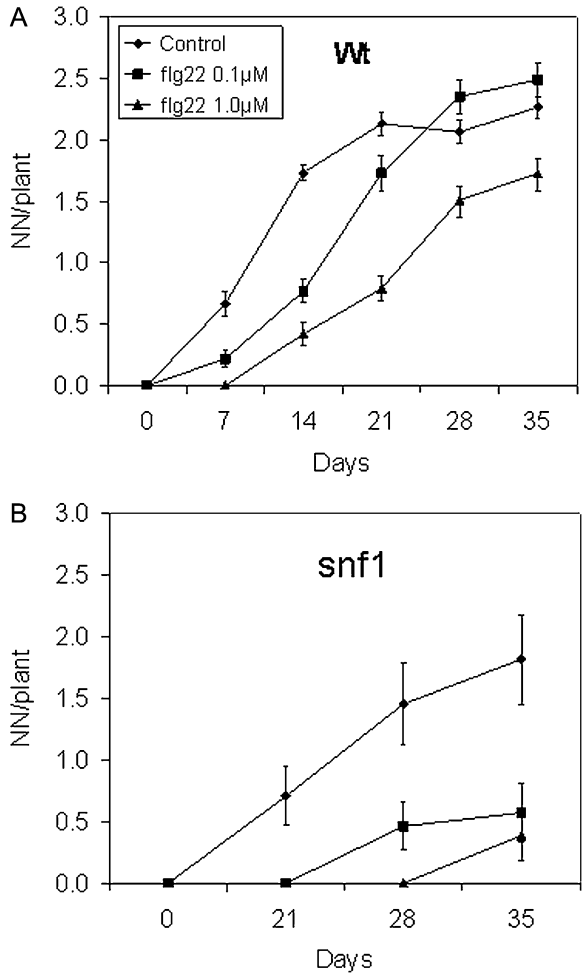

Fig. 4. Nodule number (NN) per plant in wild-type *L. japonicus* inoculated with *M. loti* and the *snf1* mutant treated with 0.1 μM and 1.0 μM flg22. Each point represents the average and SE of 30 different plants.

The negative effect of flg22 is restricted to the establishment of symbiosis

Two time-course experiments were performed in order to determine whether the negative effect of flg22 in nodule formation affects the formation of new nodules once the symbiosis is established and the nitrogen fixation process is active. In the first experiment the plants were watered with nutrient solution containing 0.1 μM flg22 and inoculated with *M. loti* simultaneously. The nodule number and nitrogenase activity were monitored from the fourth to the seventh week after inoculation. Plants treated with flg22 developed ~40% fewer nodules during the first 3 weeks, with a reduction in this difference to 20% at 7 weeks after inoculation (Fig. 6A). Nitrogenase activity per plant was also reduced ~25% in the presence of flg22, except at 7 weeks after inoculation when the difference relative to control plants was 12%, indicating a correlation between the decrease in the nodule number and the nitrogenase activity per plant. In the second experiment, *Lotus* plants were watered with 0.1 μM flg22 4 weeks after inoculation when the symbiosis was established and then the nodule number and nitrogenase activity were monitored in the following 4 weeks. In that case, significant differences in the nodule number and nitrogenase activity per plant were not detected (Fig. 6B), suggesting that once the symbiosis between *Lotus* and *M. loti* is established flg22 does not interfere with the formation of new nodules.

LjFls2 protein characteristics and gene expression

The deduced protein sequence of LjFls2 showed that it is closely related to the *Arabidopsis* FLS2 receptor (accession no. AT5G46330.1). The predicted LjFls2 protein is 1157 amino acids long with an estimated molecular mass of 126 kDa and has 53% of amino acids identical to the *Arabidopsis* FLS2 isologue (Supplementary Fig. S2 at *JXB* online). However, they share conserved blocks dispersed over the entire protein, and LjFls2 has all the characteristics of a receptor protein kinase with an intracellular kinase domain, a transmembrane domain, and an extracellular domain with 28 tandem repeats of a 24 amino acid LRR.

The expression pattern of the flg22 receptor was analysed by RT-PCR in different organs of the *Lotus* wild type and the spontaneous nodulating mutant *snf1* in order to gain a better insight into FLS2 regulation during rhizobium–legume symbiosis. The expression pattern revealed that *LjFls2* expression is suppressed in nodules of both genotypes, with a slightly but not significantly higher expression level in roots relative to leaves (Fig. 7). The fact that *LjFls2* expression was suppressed in infected and uninfected nodules of *snf1* suggests that the down-regulation of this gene is independent of the rhizobium infection, being a consequence of the induction of nodule organogenesis.

Discussion

Roots of plants in the rhizosphere are exposed to numerous molecular signals coming from pathogenic and beneficial microorganisms such as MAMPs and NFs, respectively. To differentiate between those different types of signals, plants have developed specific types of receptors, some of them with a common evolutionary origin. One example would be the LysM receptor kinases that recognize closely related compounds including the elicitor chitin (Miya *et al.*, 2007) and rhizobial NFs, activating two opposite mechanisms such as defence and symbiosis. However, some elements of the signalling pathways and the responses induced by these receptors may be common to both processes since they have evolved from a common ancestor. In that sense, activation of defence-like responses during the rhizobium–legume symbiotic interaction has been previously reported (Kouchi *et al.*, 2004; Lohar *et al.*, 2006; Moreau *et al.*, 2011). NF recognition by their specific receptors has been proposed to be directly involved in the defence gene activation (Nakagawa *et al.*, 2011). On the other hand, the diversity of PRR (pattern recognition receptor) specificities and MAMPs highlights the existence of possible rhizobial elicitors recognized by the host legume.

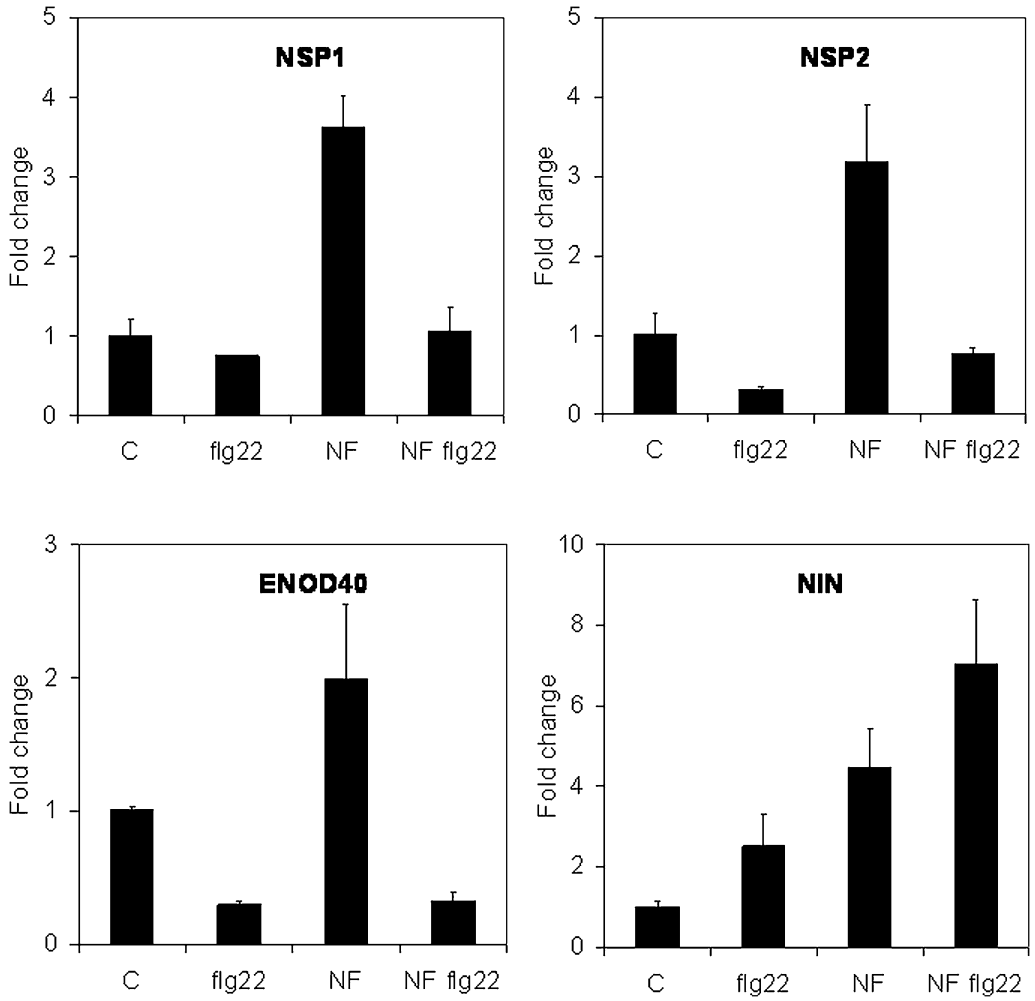

Fig. 5. Quantitative RT-PCR analysis of *NSP1*, *NSP2*, *ENOD40*, and *NIN* transcripts in roots of *L. japonicus* treated with 1.0 μM flg22 and/or NFs for 24 h. C indicates the control. Expression levels are normalized to the housekeeping gene *ATP synthase* and are presented as relative to untreated plants. The data are expressed as the means and SE of two biological replications of 10 plants.

In the present work, the defence responses induced by the well characterized elicitor flg22 and *M. loti*, the symbiotic partner of *L. japonicus*, have been compared in order to determine a possible cross-talk between defence and symbiotic signalling pathways. Both flg22 and *M. loti* induced defence responses such as ethylene accumulation (Fig. 1), MAPK activation (Fig. 2), and defence gene expression (Fig. 3), with the difference that the responses induced by the bacteria were weaker in general compared with those induced by the elicitor. Independently of the activation of some defence genes by the NF effect (Nakagawa *et al.*, 2011), these results support the possibility that the rhizobia produce some elicitors detected by the symbiotic partner. Interestingly, the broad group of rhizobial species inducing root nodules exhibits an exceptional divergence in the N-terminal conserved domain of flagellin, and peptides synthesized according to these divergent sequences proved to be completely inactive as elicitors in *Arabidopsis* and other plant species (Felix *et al.*, 1999). However, the capacity of legumes to perceive flagellin from their cognate bacteria has never been tested before. In order to rule out the possibility that flagellins from *M. loti* act as elicitors in *L. japonicus*, the defence responses induced by flg22 were compared with those induced by a suspension of flagellin purified from *M. loti*. The fact that neither ethylene accumulation nor MAPK activation was induced indicates that rhizobial flagellin is not an elicitor.

Plants have had to cope with pathogenic microorganisms since hundreds of millions of years ago, which explains the wide distribution of defence mechanisms against such pathogens in response to MAMPs. It is believed that the capacity of legumes to establish symbiotic interactions evolved from the pre-existent capacity to recognize MAMPs such as chitin, a fungal elicitor, through LysM receptor kinases that acquired the ability to perceive bacterial nodulation signals (NFs) (Zhang *et al.*, 2007). In this context, it can be inferred that FLS2 may constitute an ancient form of signalling molecule probably involved in other processes in plants that was recruited for recognition of bacteria. Therefore, the presence of a FLS2 receptor in *L. japonicus* may also precede the symbiotic capacity of this plant and consequently this must have constituted a mechanism of selection that led to the divergence of the flagellins in rhizobial bacteria. However, it is possible that during the

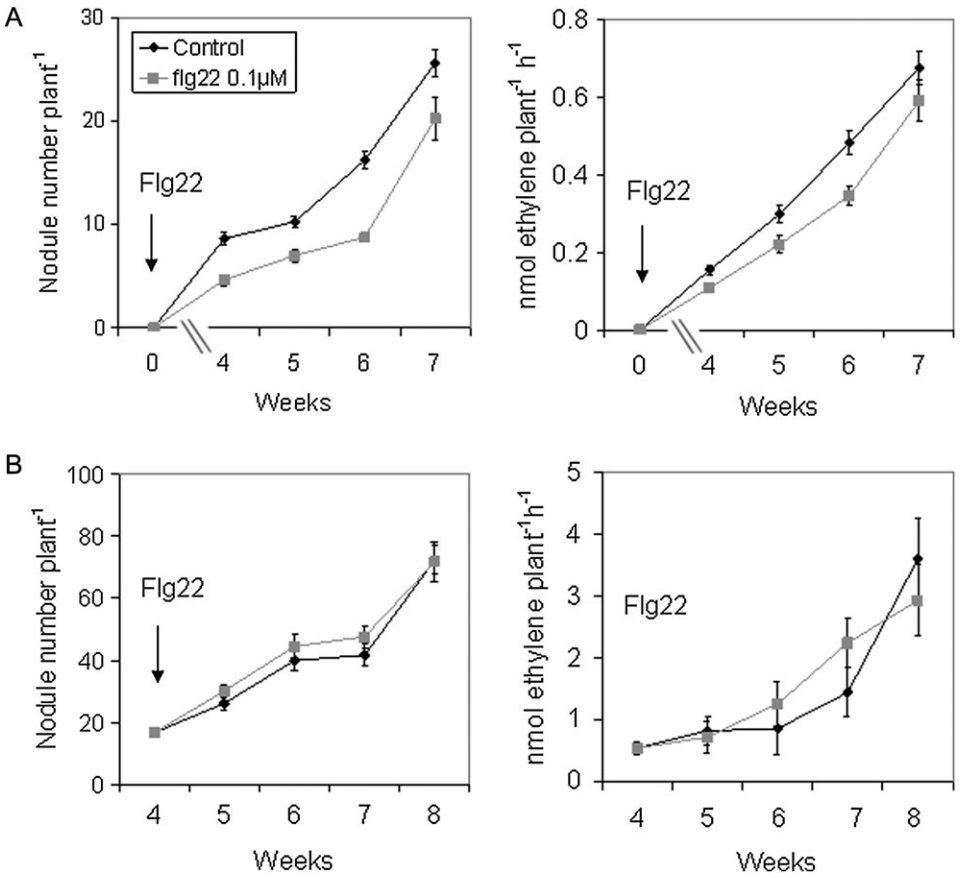

Fig. 6. (A) Nodule number and nitrogenase activity (nmol ethylene plant^{-1} h^{-1}) in *L. japonicus* plants inoculated with *M. loti* treated with 0.1 μM flg22 and untreated (Control) at the time of inoculation. (B) Nodule number and nitrogenase activity (nmol ethylene plant^{-1} h^{-1}) in *L. japonicus* plants inoculated with *M. loti* treated with 0.1 μM flg22 and untreated (Control) 4 weeks after the time of inoculation. Each point represents the average and SE of 12 plants.

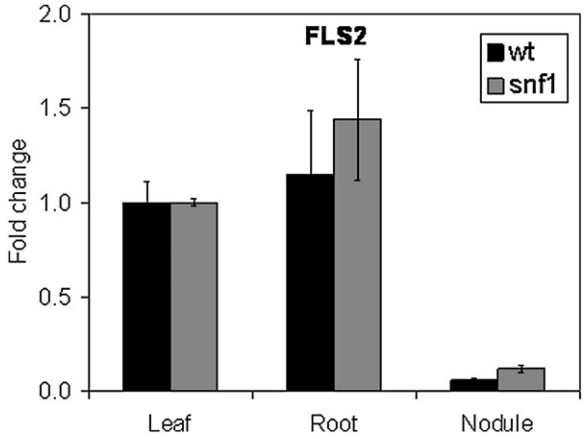

Fig. 7. Quantitative RT-PCR analysis of *LjFLS2* gene expression in leaves, roots, and nodules of *L. japonicus* wild type and *snf1*. Expression levels are normalized to the housekeeping gene *ATP synthase* and are presented as relative to leaves. The data are expressed as the means and SE of three biological replications of three plants.

evolution from an ancient intercellular infection mechanism, independent of NFs, to more recent infection mechanisms dependent on NFs (Madsen *et al.*, 2010), the rhizobia might have acquired the capacity to suppress defence responses in the nodules, as shown by the expression analysis of the FLS2 receptor in different organs of *L. japonicus* (Fig. 7). The expression level of *LjFls2* was down-regulated in the nodule tissue of infected wild-type nodules as well as in non-infected nodules of the spontaneous nodulating mutant *snf1*. This is consistent with the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) (Høgslung *et al.*, 2009) where, in addition to *LjFls2*, a down-regulation of *LjBak1* in nodules is also shown. On the basis of this finding, it is speculated that the down-regulation of *LjFls2* in nodules is activated by the NF signalling downstream of the CCaMK.

Despite the co-existence of defence and symbiotic responses in *L. japonicus*, and the possible implication of some of the defence mechanisms in the symbiotic establishment (D'Haaze *et al.*, 2003), defence responses induced by flg22 caused a negative effect in the symbiotic interaction between *L. japonicus* and *M. loti*. This negative effect resulted in a delay of nodulation (Fig. 4) that affected nodule organogenesis, as demonstrated by the negative effect of flg22 on the spontaneous nodulation of the *snf1* mutant, and the infection process, as demonstrated by the inhibition of *NSP1* and *NSP2* genes, both required for this process (Madsen *et al.*, 2010). However, the fact that flg22

did not affect the formation of new nodules when the symbiosis was already established (Fig. 6B) suggests that once the bacteria are colonizing the plant roots, the symbiotic pathway has prevalence over the defensive response. Expression analysis showed a down-regulation in the presence of NFs by flg22 of the early nodulin (ENOD40) and the putative transcription factors nodulation signalling pathway 1 (NSP1) and NSP2, indispensable for nodulation in *L. japonicus* (Heckmann *et al.*, 2006; Murakami *et al.*, 2006) (Fig. 5). These results confirm the antagonistic effect of the defence pathway on nodule primordia formation, and are in agreement with the nodulation delay observed in the nodulation kinetics (Fig. 4). On the other hand, the transcriptional regulator nodule inception (NIN), which is involved in infection thread formation as well as nodule primordia initiation (Schauser *et al.*, 1999), behaved in a different way and was up-regulated by flg22 (Fig. 5). NIN seems to be involved in the negative regulation of root cell competence in the invasion zone in *L. japonicus* plants since *nin* mutants displayed excessive root hair deformation with an ~4-fold expansion of the infection zone (Schauser *et al.*, 1999). This role of NIN as a negative regulator of infection susceptibility may be behind its up-regulation by flg22 and could indicate interplay between nodulation and the defence signalling pathway.

In summary, the present results show that defence and symbiotic pathways overlap in the mutualistic interaction between *L. japonicus* and *M. loti*, most probably due to the presence of bacterial elicitors different from flagellin in the microsymbiont. It should be noted that during the establishment of symbiosis, the defence responses had prevalence over the symbiotic responses, as demonstrated by the nodulation delay and down-regulation of genes involved in nodule morphogenesis induced by flg22 (Fig. 4). However, once the symbiosis was established, treatment with flg22 did not interfere with the formation of new nodules (Fig. 6B), which implies that the effect of flg22, and therefore the plant defence response, is restricted to the early nodulation events.

Supplementary data

Figure S1. SDS–PAGE of flagellin preparation from *M. loti*.

Figure S2. Alignment of FLS2 proteins from *L. japonicus* and *Arabidopsis thaliana*.

Table S1. Primer sequences of *Lotus japonicus* genes used for quantitative real-time PCR.

Table S2. *Lotus japonicus* Fls2 cDNA clones.

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References

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415,** 977–983.

Bek AS, Sauer J, Thygesen MB, Duus JO, Petersen BO, Thirup S, James E, Jensen KJ, Stougaard J, Radutoiu S. 2010. Improved characterization of Nod factors and genetically based variation in LysM receptor domains identify amino acids expendable for Nod factor recognition in *Lotus* spp. *Molecular Plant-Microbe Interaction* **23,** 58–66.

Boller T, Felix G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* **60,** 379–406.

Broughton WJ, Dilworth MJ. 1971. Control of leghaemoglobin synthesis in snake beans. *Biochemical Journal* **125,** 1075–1080.

Cárdenas L, Martinez A, Sanchez F, Quinto C. 2008. Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). *The Plant Journal* **56,** 802–813.

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JD, Felix G, Boller T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448,** 497–500.

Colcombet J, Hirt H. 2008. Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. *Biochemical Journal* **413,** 217–226.

Cullimore JV, Ranjeva R, Bono JJ. 2001. Perception of lipo-chitooligosaccharidic Nod factors in legumes. *Trends in Plant Science* **6,** 24–30.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference gnes for transcript normalization in Arabidopsis. *Plant Physiology* **139,** 5–17.

D'Haeze W, De Rycke R, Mathis R, Goormachtig S, Pagnotta S, Verplancke C. 2003. Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. *Proceedings of the National Academy of Sciences, USA* **100,** 11789–11794.

Felix G, Duran JD, Volko S, Boller T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant Journal* **18,** 265–276.

Gómez-Gómez L, Boller T. 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell* **5,** 1003–1011.

Handberg K, Stougaard J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *The Plant Journal* **2,** 487–496.

Hann DR, Rathjen JP. 2007. Early events in the pathogenicity of *Pseudomonas syringae* on *Nicotiana benthamiana*. *The Plant Journal* **49,** 607–618.

Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, Parniske M, Wang TL, Downie JA. 2006. *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is

functionally conserved in a non-legume. *Plant Physiology* **142,** 1739–1750.

Høgslund N, Radutoiu S, Krusell L, *et al*. 2009. Dissection of symbiosis and organ development by integrated transcriptome analysis of *Lotus japonicus* mutant and wild-type plants. *PLoS One* **4,** e6556.

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ, Kumagai H. 2004. Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. *DNA Research* **11,** 263–274.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. *Methods* **25,** 402–408.

Lohar DP, Sharopova N, Endre G, Peñuela S, Samac D, Town C, Silverstein KAT, VandenBosh KA. 2006. Transcript analysis of early nodulation events in *Medicago truncatula*. *Plant Physiology* **140,** 221–234.

Mackey D, McFall AJ. 2006. MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Molecular Microbiology* **61,** 1365–1371.

Madsen EB, Madsen LH, Radutoiu S. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* **425,** 637–640.

Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronson CW, James EK, Stougaard J. 2010. The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. *Nature Comunications* **1,** 10.

Minami E, Kouchi H, Cohn JR, Ogawa T, Stacey G. 1996. Expression of the early nodulin, ENOD40, in soybean roots in response to various lipochitin signal molecules. *The Plant Journal* **10,** 23–32.

Moreau S, Verdenaud M, Ott T, Letort S, de Billy F, Niebel A, Gouzy J, de Cavalho-Niebel F, Gamas P. 2011. Transcriptional reprogramming during root nodule development in *Medicago truncatula*. *PLoS One* **6,** e16463.

Murakami Y, Miwa H, Imaizumi-Anraku H, Kouchi H, Downie JA, Kawaguchi M, Kawasaki S. 2006. Positional cloning identifies *Lotus japonicus* NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. *DNA Research* **13,** 255–265.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **104,** 19613–19618.

Nafisi M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA, Glazebrook J. 2007. Arabidopis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexyn synthesis. *The Plant Cell* **19,** 2039–2052.

Nakagawa T, Kaku H, Shimoda Y, Sugiyama A, Shimamura M, Takanashi K, Yazaki K, Aoki T, Shibuya N, Kouchi H. 2011. From defense to symbiosis: limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume–Rhizobium symbiosis. *The Plant Journal* **65,** 169–180.

Nicaise V, Roux M, Zipfel C. 2009. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiology* **150,** 1638–1647.

Niwa S, Kawaguchi M, Imazumi-Anraku H, Chechetka SA, Ishizaka M, Ikuta A, Kouchi H. 2001. Responses of a model legume *Lotus japonicus* to lipochitin oligosaccharide nodulation factors purified from *Mesorhizobium loti* JRL501. *Molecular Plant-Microbe Interaction* **14,** 848–856.

Perret X, Staehelin C, Broughton WJ. 2000. Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* **64,** 180–201.

Radutoiu S, Madsen LH, Madsen EB, *et al*. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425,** 585–592.

Radutoiu S, Madsen LH, Madsen EB, Jurkiewicz A, Fukai E, Quistgaard EM, Albrektsen AS, James EK, Thirup S, Stougaard J. 2007. LysM domains mediate lipochitin–oligosaccharide recognition and Nfr genes extend the symbiotic host range. *EMBO Journal* **26,** 3923–3935.

Ramu SK, Peng HM, Cook DR. 2002. Nod factor induction of reactive oxygen species production is correlated with the expression of the early nodulin gene *rip1 Medicago truncatula. Molecular Plant-Microbe Interaction* **15,** 522–528.

Robatzek S, Bittel P, Chinchilla D, Kochner P, Felix G, Shiu SH, Boller T. 2007. Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities. *Plant Molecular Biology* **64,** 539–547.

Schauser L, Roussis A, Stiller J, Stougaard J. 1999. A plant regulator controlling development of symbiotic root nodules. *Nature* **402,** 191–195.

Sun W, Dunning FM, Pfund C, Weingarten R, Bent AF. 2006. Within species flagellin polymorphism in *Xanthomonas campestris pv* campestris and its impact on elicitation of Arabidopsis FLAGELLIN SENSING2-dependent defenses. *The Plant Cell* **18,** 764–779.

Takai R, Isogai A, Takayama S, Che FS. 2008. Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. *Molecular Plant-Microbe Interaction* **21,** 1635–1642.

Tirichine L, Imaizumi-Anraku H, Yoshida S, *et al*. 2006*a*. Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* **441,** 1153–1156.

Tirichine L, James EK, Sandal N, Stougaard J. 2006*b*. Spontaneous root-nodule formation in the model legume *Lotus japonicus*: a novel class of mutants nodulates in the absence of Rhizobia. *Molecular Plant-Microbe Interactions* **19,** 373–382.

Zhang XC, Wu X, Findley S, Wan J, Libault M, Nguyen HT, Cannon SB, Stacey G. 2007. Molecular evolution of lysin motif-type receptor-like kinases in plants. *Plant Physiology* **144,** 623–636.